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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. §1.53(b)(2)

Atty. Docket: LEVI-SCHAFFER1

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TITLE OF THE INVENTION (280 characters max)

IRp60 IS EXPRESSED AND FUNCTIONAL ON HUMAN CORD BLOOD DERIVED MAST CELLS

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ENCLOSED APPLICATION PARTS (check all that apply)

[x] Specification	Number of Pages	11	[x] Applicant claims small entity status. See 37 C.F.R. §1.27
[x] Drawing(s)	Number of Sheets	16	[ ] Other (specify) _____

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[X] Credit Card Payment Form PTO-2038 is enclosed to cover the Provisional filing fee of

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The invention was made by an agency of the United Stated Government or under a contract with an agency of the United States Government.

[X] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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1. Topic of proposed research

The research proposed herein focuses on a novel approach for an immunopharmacological modulation of effector-cell function in allergic inflammation as a preventive and therapeutic tool. Our aim is to generate a construct termed BiscFv [bifunctional single-chain Fv construct], targeted to a mast cell inhibitory receptor recently found and characterized in our laboratory and to a specific cell surface marker. The BiscFv is therefore designed to selectively activate this inhibitory mechanism on mast cells, theoretically eliciting a widespread negative effect on their survival and function in allergic-inflammatory settings such as asthma, allergic conjunctivitis, rhinitis, atopic eczema etc.

2. Brief description of the subject and of the scientific and technological background

A. Allergic inflammation

Allergic inflammation is a complex phenomenon, involving various cell types such as inflammatory and structural cells. Mast cells are the well-established initiators of allergic inflammation, attracting, activating and finally interacting with other inflammatory cells, mainly the eosinophils. Allergic inflammation comprises a variety of pathologies, such as asthma, allergic rhinitis, allergic conjunctivitis, atopic eczema etc. Among these diseases, and as an example, asthma, is the most common illness of early childhood, counting for up to 20% in Western countries and currently increasing [1].

Experimentation in the field of allergy has provided insights into the cellular and molecular mechanisms underlying these pathologies. These investigations have led to the understanding that the allergic response is often biphasic. The first, early phase is initiated by **mast cell** activation (see mast cells), while the second, late phase is brought about by the infiltration of inflammatory cells, predominantly T-cells, eosinophils and some basophils [2]. Knowledge, however, has not yet yielded efficacious therapeutic means. Currently used approaches offer either symptomatic relief (i.e. anti-histamines and anti-leukotrienes) or a non-selective anti-inflammatory treatment (i.e. glucocorticosteroids). In addition, newly developed immunopharmacological treatments targeting a single T cell cytokine (i.e. anti-IL-5) or transcription factors (i.e. STAT-6, GATA-3 or FOG-1) have not proven efficient as yet. An additional drawback concerning currently available mAb based therapy is that it can be administered to the patients only by intravenous administration.

B. Mast cells

Mast cells are tissue dwelling, FcεRI bearing cells containing prominent cytoplasmic granules. Besides having a pivotal role in allergic reactions, they are also involved in fibrosis, tumors, autoimmune diseases and innate immunity. Mast cells are widely distributed throughout the body, in connective tissues and on mucosal surfaces where they are usually located in close proximity to blood vessels and peripheral nerves. Therefore, they are exposed to environmental stimuli such as microorganisms and allergens with which they can react, both within minutes and/or over a period of hours, and undergo regulated secretion of preformed and newly synthesized mediators.

Upon activation, mast cells release a variety of inflammatory mediators including pre-formed granule constituents (e.g. histamine, proteoglycans and proteases), PGD_2, LTC_4, PAF, and to a lesser extent, LTB_4, and a variety of cytokines (e.g. IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, RANTES, IFN-γ, TGF-β, TNF-α, and GM-CSF)[3].

In addition to the classical "allergic" IgE-dependent mast cell activation that is triggered by the binding of allergens to two adjacent IgE molecules bound to FcεRI, there are other ways of mast cell stimulation. IgE-independent mast cell activation may be particularly important in the setting of the late phase and in chronic inflammation. Notably, while anti-IgE therapy is now approved for the

treatment of asthma, it only induces a modest improvement. This highlights the involvement of non-IgE dependent pathways in the development of asthma as well as the need for new targets for therapeutic intervention. Indeed, work done in our laboratory (as well as in others) has shown that numerous mediators are capable of activating mast cells. Among them, stem cell factor (SCF), that is critically responsible for mast cell differentiation, survival, proliferation, maturation, chemotaxis, adhesion, and activation as well as Nerve Growth Factor (NGF) that also induces mast cell activation.
The IgE-independent stimulation of mast cells can also be triggered by polybasic compounds that share similar structural features essential for their activity such as compound 48/80, neuropeptides (VIP, CGRP, substance P, neurotensin), and eosinophil derived-major basic protein (MBP) [4].

Eosinophils encounter mast cells in the tissue during the late phase of the allergic inflammatory process. Recently, evidence has emerged indicating an important cross-talk between these two cells exists. Work done in our laboratory has shown that eosinophil survival is enhanced by mast cell-derived TNF-α via TNF-αRI and TNF-αRII. Furthermore, the preformed mast cell-derived tryptase induces IL-6 and IL-8 production and release from human peripheral blood eosinophils by PAR-II initiating the mitogen-activated protein kinase/AP-1 pathway, while GM-CSF produced by IgE-activated mast cells induces eosinophil survival and eosinophil cationic protein (ECP) release. Human lung-derived mast cells become responsive to MBP when co-cultured with fibroblasts, by a process dependent on membrane-bound SCF. Notably, eosinophils also synthesize SCF and NGF,. Altogether, all this strengthens the importance of mast cells in the late and chronic stages of allergic inflammation [5-6].

It has recently become clear that mast cell degranulation is regulated by additional surface activatory and inhibitory receptors such as FcγRIIB, gp49A/B1/B2, PIR-B, LIRs/ILTs and sialic acid binding Ig-like lectins (siglecs) that are expressed on mast cells and functional at least on murine mast cells [7]. In addition, recently we have demonstrated that human cord blood mast cells express the IRp60 inhibitory receptor (see **Inhibitory Receptors** and **Figure 1A, 1B**). Moreover, both lung-derived and cord blood-derived mast cells express the inhibitory receptors p75/AIRM and FcγRIIB (data not shown).

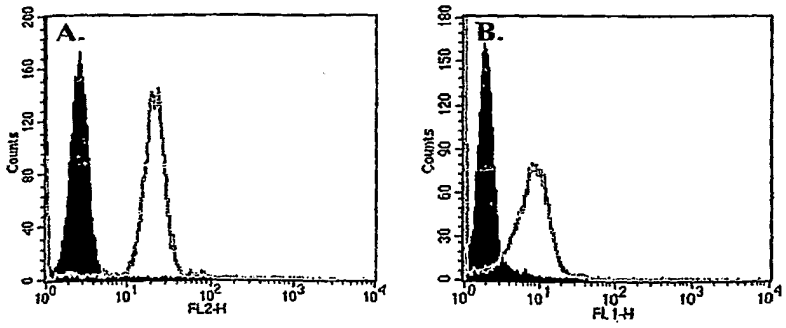


Figure 1: FACS analysis of IRp60 expression on human cord blood-derived mast cells (A) and human lung mast cells (B). Cells were incubated with anti-IRp60 mAb (1:10 dilution, 30min, on ice) followed by goat anti-mouse PE or FITC (1:200 dilution, 30min, on ice).
Human cord blood-derived mast cells express high levels of IRp60 (A) (n=5) Human lung mast cells express IRp60 as well (B) (n=3). Filled histograms and blank histograms represent isotype control and IRp60 staining, respectively.

It is noteworthy, that most of the knowledge on mast cells to date relies on studies performed on on rodent mast cells and human mast cell lines. In our laboratory, for our interest in human therapy, we have developed during the years the technology to purify and culture specifically human mast cells from cord blood, lung, skin and intestine.

C. Inhibitory Receptors

Over the past several years it has become increasingly apparent that both mast cells and eosinophils express several inhibitory receptors belonging either to the Ig receptor superfamily (characterized by a single V-type Ig-like domain in the extracellular portion such as KIRs, LIRs/ILTs, LAIR, gp49B1 etc. or to the c-type (calcium dependent) lectin superfamily (such as MAFA, CD94/NKG2A). This large family of immune inhibitory receptors can be identified by a consensus amino acid sequence, the immunoreceptor tyrosine-based inhibitory motif (ITIM). The ITIM is present in the cytolplasmic domain of these molecules. The archetype ITIM sequence is composed of 6 amino acids (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val), where X denotes any amino acid. Upon activation, these inhibitory receptors undergo tyrosine phosphorylation, often by a Src family kinase, which provides a docking site for the recruitment of cytoplasmic phosphatases having a Src homology 2 (SH2) domain such as SHP-1,-2 and SHIP-1,-2 [8].

As previously described mast cells can be activated by IgE-dependent (FcεRI mediated) and IgE-independent stimuli. Activation of mast cells via IgE-dependent mechanisms results in rapid recruitment of syk and lyn to tyrosine phosphorylated residues in the intracellular component of the FcεRI receptor termed ITAM (immunoreceptor tyrosine-based activatory motif). The consequence of this action is histamine and other preformed mediators release and synthesis and release of lipid mediators by a rapid process that is completed in less than 30 min. In addition, SCF and NGF that activate mast cells, are dependant on Src family kinases. Interestingly both IgE-dependent and independent stimuli are regulated by inhibitory receptors at least *in-vivo* in mice models. Thus, recruitment of SHP-1, -2 and SHIP-1,-2 that dephosphorylate ITAM domains or kinase activity result in downregulation of mast cell activation. This inhibition has been thoroughly described for the gp49B1 inhibitory receptor on murine mast cells, where co-ligation of the inhibitory receptor with FcεRI resulted in inhibition of secretory granule mediator (histamine, β-hexosaminidase) and LTC_4 release [9].

IRp60 is an inhibitory receptor belonging to the Ig superfamily. It is expressed on many cell types such as T-cells, NK cells and granulocytes. Cross-linking of IRp60 on NK cells, results in down-regulation of NK cytolyitic activity. In addition, treatment of IRp60 with sodium pervanadate led to marked IRp60 tyrosine phosphorylation and association with both SHP-1 and SHP-2 [10]. Furthermore, IRp60 cross-linking inhibited the cytolitic activity of T-cell clones in redirected killing assays using anti-CD3 mAb. Importantly, the ligand of IRp60 is yet unknown.

A recent study assessing the role of SHP-1 inhibitory signaling in Th1/Th2 cell differentiation and in the development of Th2-dependent allergic airway inflammation was performed in natural SHP-1 mutant mice (*me/+* mice). In this study it has has been observed that SHP-1 controls the development of OVA-induced allergic airway inflammation. In fact AHR, peri-bronchial and peri-vascular inflammation and eosinophil infiltration was enhanced in bronchoalveolar lavage fluid of *me/+* mice when compared with w.t mice [11].

Most importantly to our proposal, we have recently shown that IRp60 expressed on human mast cells is functional. Cross-linking of IRp60 inhibits both β-hexosaminidase and IL-4 release in response to IgE-dependent activation (see **Fig. 2A, 2B**).

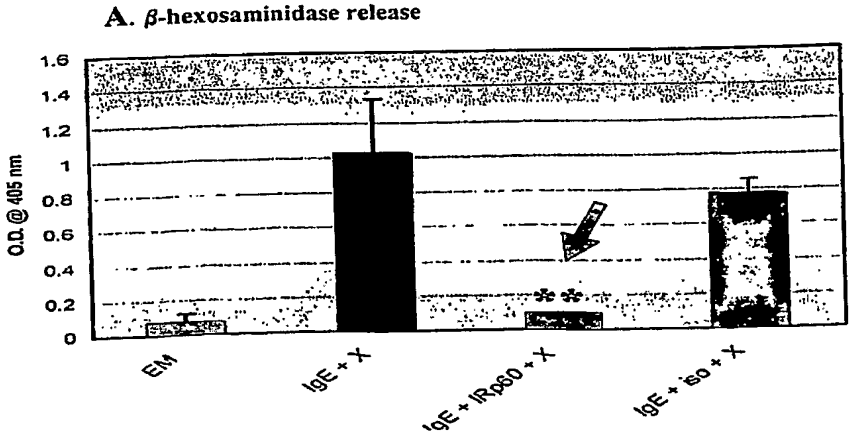
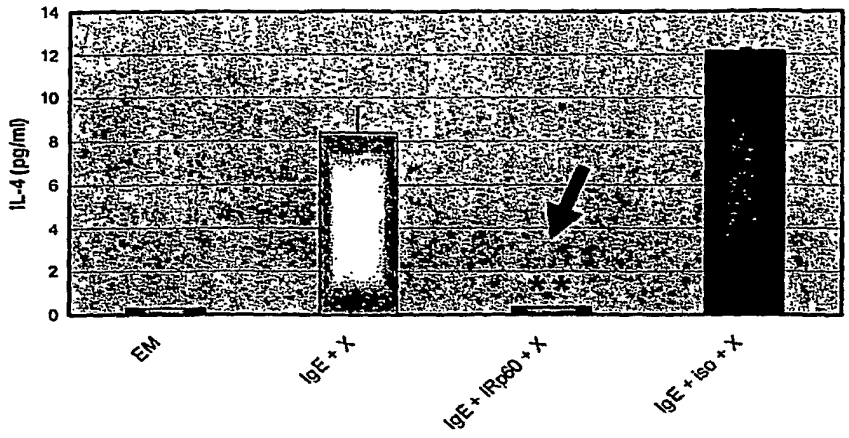


Figure 2: Mast cells were incubated 5 days prior to activation with chimeric (human Fc, murine Fab) IgE anti-NP (5μg/ml). On the day of activation, the cells were washed, and seeded onto 96 multi-well dish pre-coated with sheep anti-mouse (X) (25μg/ml) and an antibody recognizing IRp60 (3:7 dilution) or isotype matched control (iso). At the same time, another cross-linker is added (goat anti-mouse lambda-chain specific, 5μg/ml), and the plate was then incubated for 1hr. IRp60 inhibits IgE-dependent β-hexosaminidase release (A) and IL-4 release (B). The data shown is a representative of one experiment out of 3.

B. IL-4 release



D. Bi-functional single chain Fv (BiscFv).

In recent years, antibody therapy has become a new treatment modality for a vast array of diseases such as cancer, malaria and asthma. Despite this fact, it is widely agreed that the efficacy of antibodies requires further improvement. Bispecific antibodies are proteins that have 2 different binding specifities, usually designed to recognize two different antigens on different cells. The BiscFv technology has been studied in the context of immune regulation mostly in cancer and parasitc settings [12]. Thus, one binding site is specific for an antigen on the target cell (i.e. infected or cancer cell) while the other binding site recognizes specifically an antigen on the immune effector cell. Accordingly, the effector-cell mechanisms will be exerted upon the target cell leading to an appropriate immune response.

Many different types of Bispecific molecules have been designed. First-generation bispecific antibodies were produced by fusing two established hybridoma cell lines to form quadromas [13] or by chemical cross-linking of respective F(ab') fragments [14]. Both *in-vitro, in-vivo* and clinical studies done with such bispecific antibodies confirmed the therapuetical potential of such a treatment [15]. However, these studies also highlighted some predictable problems associated with this generation of antibodies. Since these antibodies contain also a murine component, immunogenicity towards the treatment developed in most patients, hence preventing retreatment with these antibodies. Advances in protein engineering technology have led to design new and less immunogenic bispecific antibodies. Bispecific single-chain Fv (BiscFv) fragments have been generated using a polypeptide linker that allows the construct to maintain its designated flexibility and function. These BiscFv antibodies are expressed as single covalently linked molecules, showing high thermal stability [16]. To date the vast majority of BiscFv constructs have been designed in cancer settings. Therefore, Fv fragments derived from monoclonal antibodies against a variety of tumor antigens were combined with single chain Fv fragments directed to recruite effector T and NK cells. Interestingly a recombinant CD3-CD19 BiscFv was highly effective in redirecting T cells against CD19-positive lymphoma cells [17] and was the first BiscFv to enter clinical trials.

In our proposal we aim to generate a BiscFv designed to recognize the inhibitory receptor IRp60 and FcεRI-bound IgE both expressed on the mast cell surface. Since in allergic patients IgE are bound to FcεRI on mast cells, a "smart" way for targeting the construct towards these cells will be achieved by designing one arm of the BiscFv towards this specific molecule (see **Fig. 3**). In addition, basophils are the only other cells expressing high levels of FcεRI on their surface thus, it is conceivable that this cell tyope will be targeted as well. The BiscFv is currently being generated using a common and well based protein engineering technology protocol (see methods). In fact we have already established several *in-vitro* results confirming our hypothesis that targeting inhibitory

receptors can decrease mediator release from mast cells ((see **Fig. 2A, 2B).** Furthermore, the hybridomas that produce mAb recognizing IRp60, which constitute the greatest limiting factor are already in our possession.

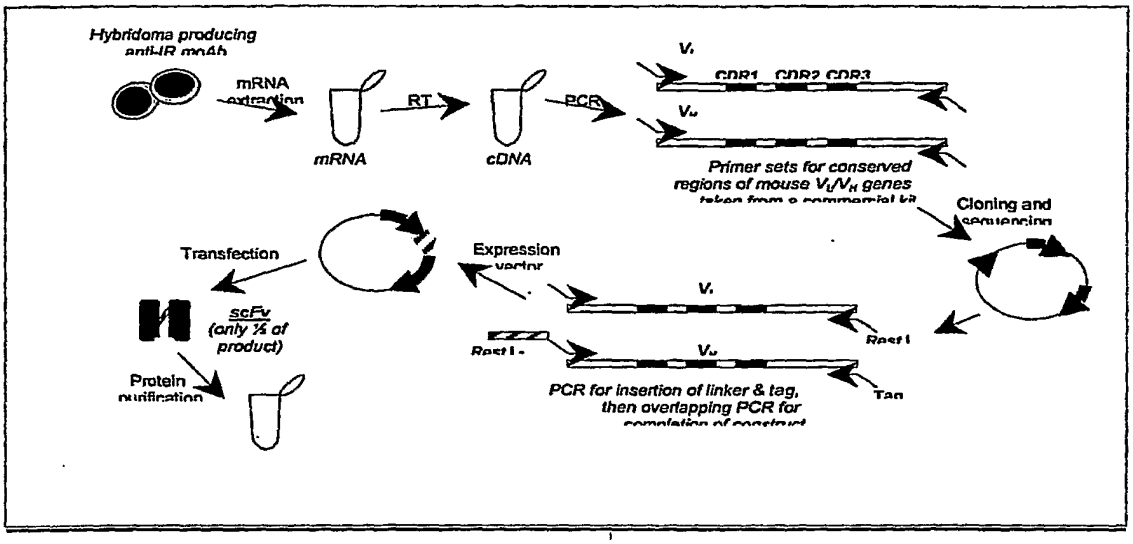


Figure 3: A schematic diagram of the designated procedure for BiscFv generation. mRNA will be extracted from hybridoma cell lines generating IRp60, IgE or isotype matched control. The V_L/V_H conserved regions will be amplified , cloned and sequenced using a commercial kit. Four sets of primers will be designed according to the sequences, containing also the DNA encoding for the $(Gly_3Ser)_4$ motif-based flexible region linker, a tag for protein purification purposes, or a restriction enzyme site. A series of overlapping PCRs will be performed to yield the desired BiscFv construct, and then using the restriction enzyme sites inserted in the flanking primers, the product will be cloned into a linearized baculovirus expression vector. The vector will be transformed into an expression system and conditions will be calibrated in order to achieve optimal yield. The BiscFv protein product will be purified on an affinity column using the PCR-inserted tag.

3. Aims

A. **Specific Aim 1:** To generate a bifunctional single chain Fv (BiscFv) recognizing both IRp60 and IgE on human mast cells.
B. **Specific Aim 2:** To test and evaluate the efficiency of the BiscFv reagent in-vitro as a platform for future clinical studies.

4. Detailed plan and methodology

Specific Aim 1: To generate a bifunctional single chain Fv (BiscFv) recognizing both IRp60 and IgE on human mast cells (see .

1. Total mRNA will be extracted from hybridoma cell lines producing immunoglobulins specific to either IRp60 or IgE. In addition, mRNA will be extracted from a hybridoma recognizing a non-related epitope. This epitope will be used in combination with an inhibitory receptor, an effector-cell marker, or itself as a specificity control for *in-vitro/ in-vivo* studies, and also as a placebo group for future clinical tests.
Most of these hybridoma species are already in our possession, while the others are in the process of generation.

(In the following stages, IRp60 related genes are tagged I and IgE related genes are tagged E)

2. Reverse transcription using SuperScript™ kit will be employed according to the manufacturer's instructions in order to yield cDNA.
3. PCR will be used to amplify the genes encoding the $V_L^{(I)}/V_H^{(I)}$ and $V_L^{(E)}/V_H^{(E)}$ chains using primers from the mouse Ig-prime kit (Novagen) hybridizing with the conserved regions flanking the V_L/V_H genes of both *I/E* epitopes.
4. The four products will be inserted into a simple vector and sequenced.
5. Four sets of primers will be designed according to the sequences, containing also the DNA encoding for the $(Gly_3Ser)_4$ motif-based flexible region linker, a tag for protein purification purposes, or a restriction enzyme site. The primers will be designed as follows (*x*=gene sequence; *RE*=restriction enzyme site; *L*=linker; *T*=Tag for protein purification) brought in 5' to 3' direction:

a. $V_L^{(I)}$-5': [RE]-x
b. $V_L^{(I)}$-3': x
c. $V_H^{(I)}$-5': [L]-x
d. $V_H^{(I)}$-3': x
e. $V_L^{(E)}$-5': [L]-x
f. $V_L^{(E)}$-3': x
g. $V_H^{(E)}$-5': [L]-x
h. $V_H^{(E)}$-3': [RE]-[T]-x

Construct sequence: RE—$V_L^{(I)}$—L—$V_H^{(I)}$—L—$V_L^{(E)}$—L—$V_H^{(E)}$—T—RE

6. A series of overlapping PCRs will be performed to yield the desired BiscFv construct, and then using the restriction enzyme sites inserted in the flanking primers, the product will be cloned into a linearized baculovirus expression vector.
7. The vector will be transformed into an expression system and conditions will be calibrated in order to achieve optimal yield.
8. The BiscFv protein product will be purified on an affinity column using the PCR-inserted tag.
These stages will be performed for every BiscFv in the mixture as follows: IRp60/IgE, Isotype/IgE, IRp60/Isotype, Isptype/Isotype, total of 4 BiscFv's. .

Specific Aim 2: To test and evaluate the efficiency of the BiscFv reagent in-vitro as a platform for future clinical studies.

In-vitro
For *in-vitro* studies, at least two human sources of mast cells will be employed, i.e. cord blood and lung mast cells. Cord blood will be obtained fresh (~70mL) and loaded on Ficoll-paque gradient (Amersham). Mononuclear cell layer will be washed and cultured in MEMα in the presence of human recombinant SCF (100ng/mL, Amgen), IL-6 (10ng/ml, Peprotech) and PGE_2 (3×10^{-7}M, Sigma) for 8-12 weeks. Maturity of the cells will be evaluated by FACS analysis of intracellular tryptase and chymase, and they will be used for studies when >95% are tryptase and chymase positive.
Human lung specimens from healthy looking areas of lungs will be obtained fresh from patients undergoing surgery for removal of lung cancer. Cells will be extracted from the tissue by enzymatic digestion, following separation on Percoll gradient (Sigma). Mononuclear fraction will be blocked to

avoid unspecific binding and incubated with mouse anti-human CD117 (Pharmingen), followed by incubation with anti-mouse coated magnetic beads (Dynal). Mast cells will be eluted by using a magnet and separated from the beads using DNase digestion. Mast cells will be cultured in MEMα in the presence of SCF (100ng/ml, Amgen).

In order to discern an inhibitory effect of BiscFv on mast cells, we will use the BiscFv *in-vitro*, mimicking a simultaneous receptor cross-linking system developed in our laboratory. For this assay, mast cells will be incubated 5 days prior to activation with chimeric (human Fc, murine Fab) IgE anti-NP (5μg/ml). On the activation day, cells will be washed and transferred to 96 multi-well plate and the different BiscFv's (i.e. IRp60/IgE, IRp60/isotype, isotype/IgE, isotype/isotype) will be added. For both positive and negative controls, the cells will be seeded onto 96 multi-well dish pre-coated with sheep anti-mouse (25μg/ml) and an antibody recognizing IRp60, in various ratios, on ice. At the same time, another cross-linker is added (goat anti-mouse lambda-chain specific, 5μg/ml) on ice, and the plate is then incubated for 5 minutes, 30 minutes or overnight. The following mediators are evaluated in the supernatants: tryptase using an enzymatic-colorimetric assay, histamine using a fluorometric assay, Th1/Th2 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, IFN-γ, TGF-β) using a FACS-analyzed multi-cytokine assay, PGD_2 and LTC_4 by commercial ELISA.

Alternatively, in order to discern whether BiscFv has a regulatory function on IgE-independent activation, mast cells will be cultured in the presence of either SCF (10ug/ml) or major basic protein (20ng/ml) and BiscFv for different time points (5,30min,18 hrs). the cell supernatant will be collected to assess the above described mediators.

To assess whether BiscFv exerts the inhibitory effects on mast cells via a conserved ITIM domain, cells will be incubated with IRp60/IgE specific BiscFv or isotype matched controls BiscFv (0-10 minutes, 37°C, 5% CO2). Immunoprecipitation will be done using anti-IRp60 mAb followed by protein A/G. We will immunoblot the precipitated proteins with antibody recognizing phosphorylated tyrosine residues (4G10) or recognizing SHP-1,-2 and SHIP-1,-2 (Santa Cruz Biotechnology, CA, USA). If these molecules co-immunoprecipitate with IRp60, functional ITIMs are present on these molecules on mast cells. Therefore we will determine in a time course fashion following activation (as described above) the kinetics of protein tyrosine phosphorylation and SHP-1,-2 or SHIP-1,-2 recruitment.

NOTE:
Written informed consent will be obtained from all of the volunteers involved in this study according to the guidelines established by the relevant Hospital Human Experimentation Helsinki Committee, Israel Helsinki Committee's approval requested for HLMC from Assaf Harofe Hospital. For CBMC, approval has already been obtained from Tel Hashomer Hospital. (See enclosures).

5. Applicative significance including estimated time and financial potential

The proposed BiscFv represents a novel approach for the down-modulation of mast cell effector functions in allergic inflammatory diseases such as asthma, allergic rhinitis and conjunctivitis, atopic eczema etc. where no current efficient therapy exists besides symptomatic relief or non-selective anti-inflammatory treatment. The BiscFv will be assessed for its anti-allergic potential first in vitro on isolated purified human mast cells and on later stages in-vivo both in murine models of allergy and in patients with periennial or seasonal allergic symptoms. The BiscFv has two main therapeutic and financial advantages that go side by side. First, it is not allergen specific, thus it could be used in patients allergic to more than one allergen and even if the allergen(s) is unknown as it frequently happens in clinical settings. Second, in future therapeutic uses it can be delivered topically by sprays, creams, eye and nose drops etc and not by the umbersome intravenous route.

Moreover, the BiscFv potential may even exceed the scope discussed above. Given the proper effector mechanisms on other cells, it is conceivable that other diseases such as autoimmune diseases, where pinpoint targeting of specific cell types is desired, could be treated.
Estimated time frame for generating the BiscFv is 6-9 months. In-vitro studies will take 3 months.

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IRp60 is expressed and functional on human cord blood derived mast cells

RATIONALE

Inflammatory responses coordinated by mast cells are likely to be regulated by the cross-talk between activatory and inhibitory signals. The high-affinity receptor for IgE (FcεRI) on mast cells plays a fundamental role in initiating and controlling allergic inflammation. However little is known about human mast cell inhibitory signaling. IRp60 is a 60-kDa glycoprotein expressed on NK cells, T cell subsets and monocytes. Upon Tyrosine phosphorylation, IRp60 associates with SHP-1 and SHP-2, leading to inhibition of NK cell-mediated killing. Being interested in inhibitory receptors on human mast cells, we examined the expression and function of IRp60 on human cord blood-derived mast cells (CBMC) and human lung mast cells (HLMC).

METHODS

Mature CBMC were obtained from cord blood mononuclear cells cultured with Stem Cell Factor, IL-6 and PGE2 for 9-12 weeks. HLMC were purified from lung specimens using enzymatic digestion followed by ficol gradient and positive selection. Expression of IRp60 was assessed by FACS and Western blot. CBMC were cultured for 5 days in 96 well plate with chimeric IgE (2μg/ml). Cells were washed and transferred to 96 well plate pre-coated with cross-linker (sheep anti-mouse, 25μg/ml) and mouse anti-human IRp60 (P192), followed by addition of goat anti-mouse λ-chain (5μg/ml) (30 minutes, 37°C). β-hexosaminidase release was determined by enzymatic-colorimetric assay; IL-4 by ELISA. CBMC were incubated with eosinophil-derived major basic protein (MBP) (10-100nM) or with poly-L-arginine (25-100nM) for 12-24 hrs and IRp60 expression was assessed. Data is presented as [Mean] ± [S.D.]. Statistical analysis was done using Windows Excel two-tailed t-test assuming equal variances.

RESULTS

Human CBMC (94.5%, n=5) and HLMC (80.5%, n=3) express IRp60. Cross-linking of IRp60 together with FcεRI inhibited β-hexosaminidase release (59.28±9.55% inhibition, n=4, $p<0.001$) and IL-4 release (64.43±2.91% inhibition, n=3, $p<0.01$). Incubation with MBP for 24 hours (10 and 100mM) down-regulated IRp60 expression on CBMC (ΔMFI=11±1, n=2 for each concentration, $p<0.001$).

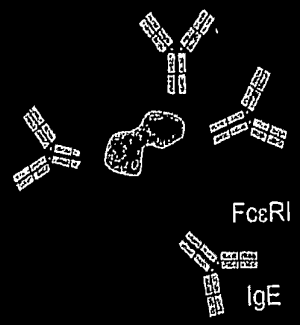
CONCLUSIONS

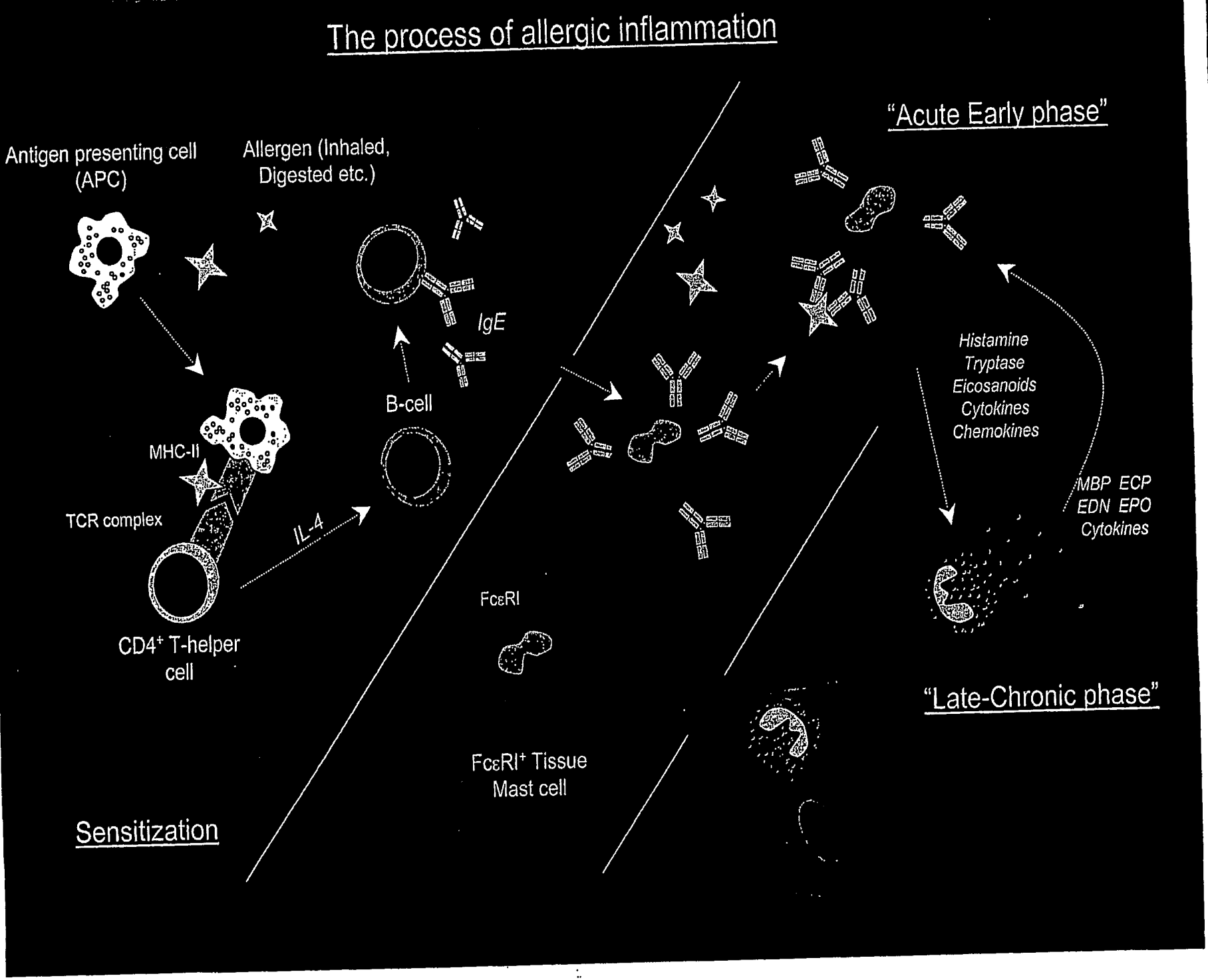
The demonstration that human mast cells express a functional IRp60 receptor suggests a novel pathway for the regulation of IgE-mediated responses.

Multitarget Single-Chain Antibody Cocktail

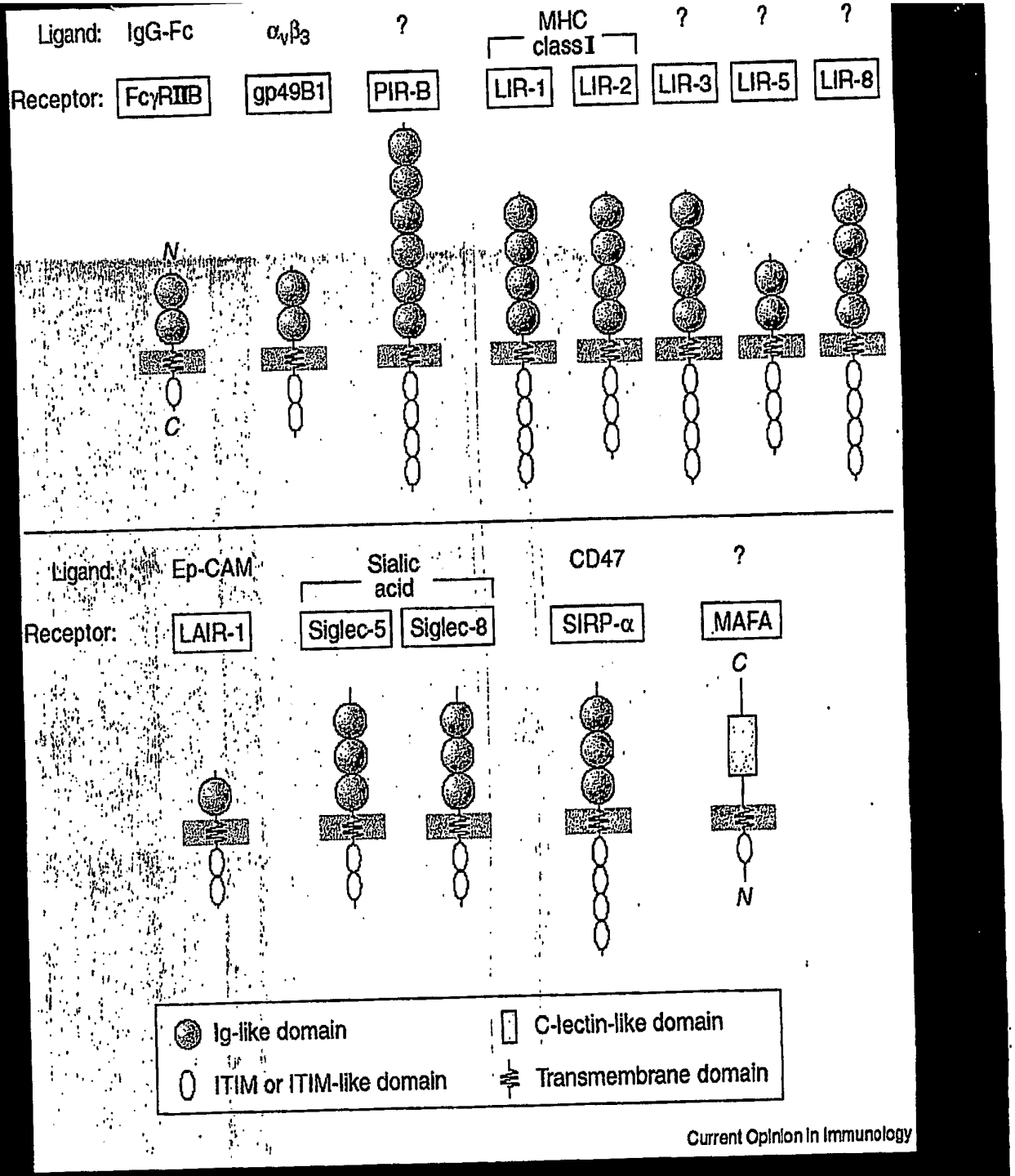
A Novel Immunopharmacological Approach for Targeting Inhibitory Receptors on Mast cells and Eosinophils as a Therapeutic Tool in Allergic Inflammation

Mast cells and eosinophils

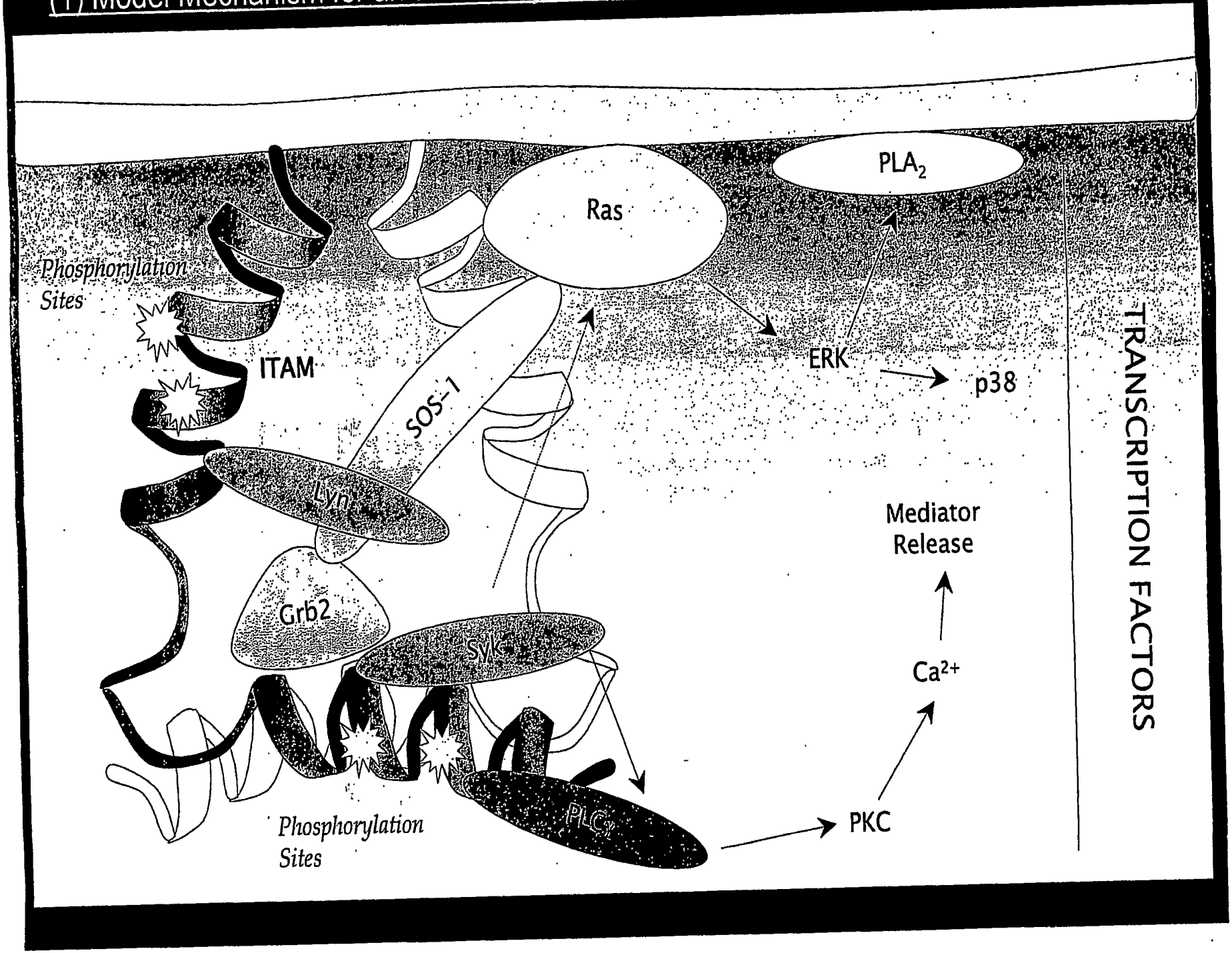
		
Growth factors	*SCF (Stem Cell Factor)* *IgE* *NGF (Nerve Growth Factor)*	*GM-CSF (Granulocyte-Monocyte Colony Stimulating Factor)* *IL-3* *IL-5*
Activatory stimuli	*IgE crosslinking by allergen Eosinophil Major Basic Protein (MBP) SCF, IL-4*	*GM-CSF* *IL-5,* *IL-3* *Secretory IgA* *C5a*
Released Mediators	*Histamine* *Tryptase* *Chymase* *Eicosanoids* *Cytokines* *Chemokines*	*Cytotoxic peptides (MBP, ECP, EDN, EPO)* *Cytokines* *Chemokines* *Eicosanoids*



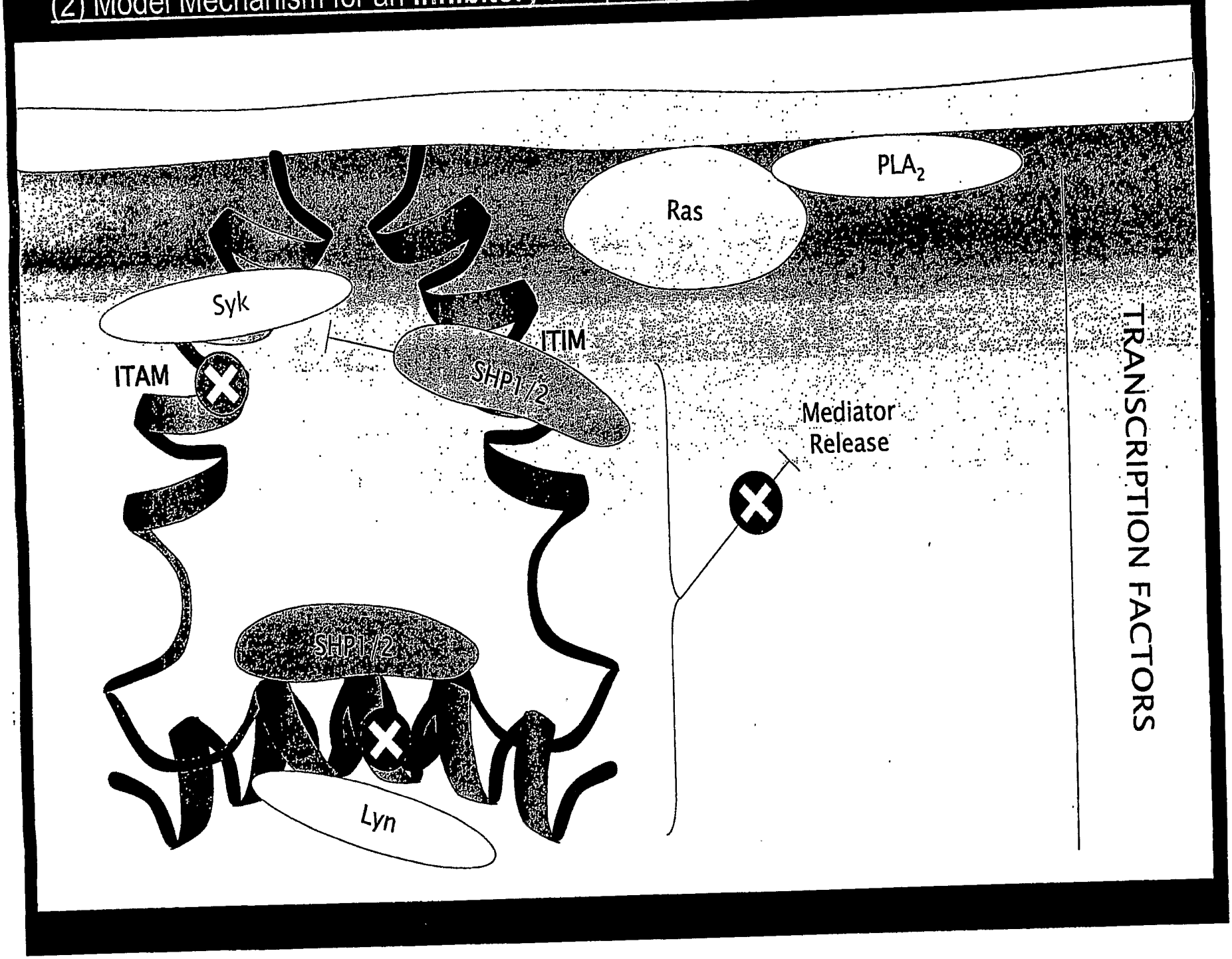
Inhibitory receptors



(1) Model Mechanism for an **activatory** receptor (FcεRI)



(2) Model Mechanism for an **inhibitory** receptor (IRp60)



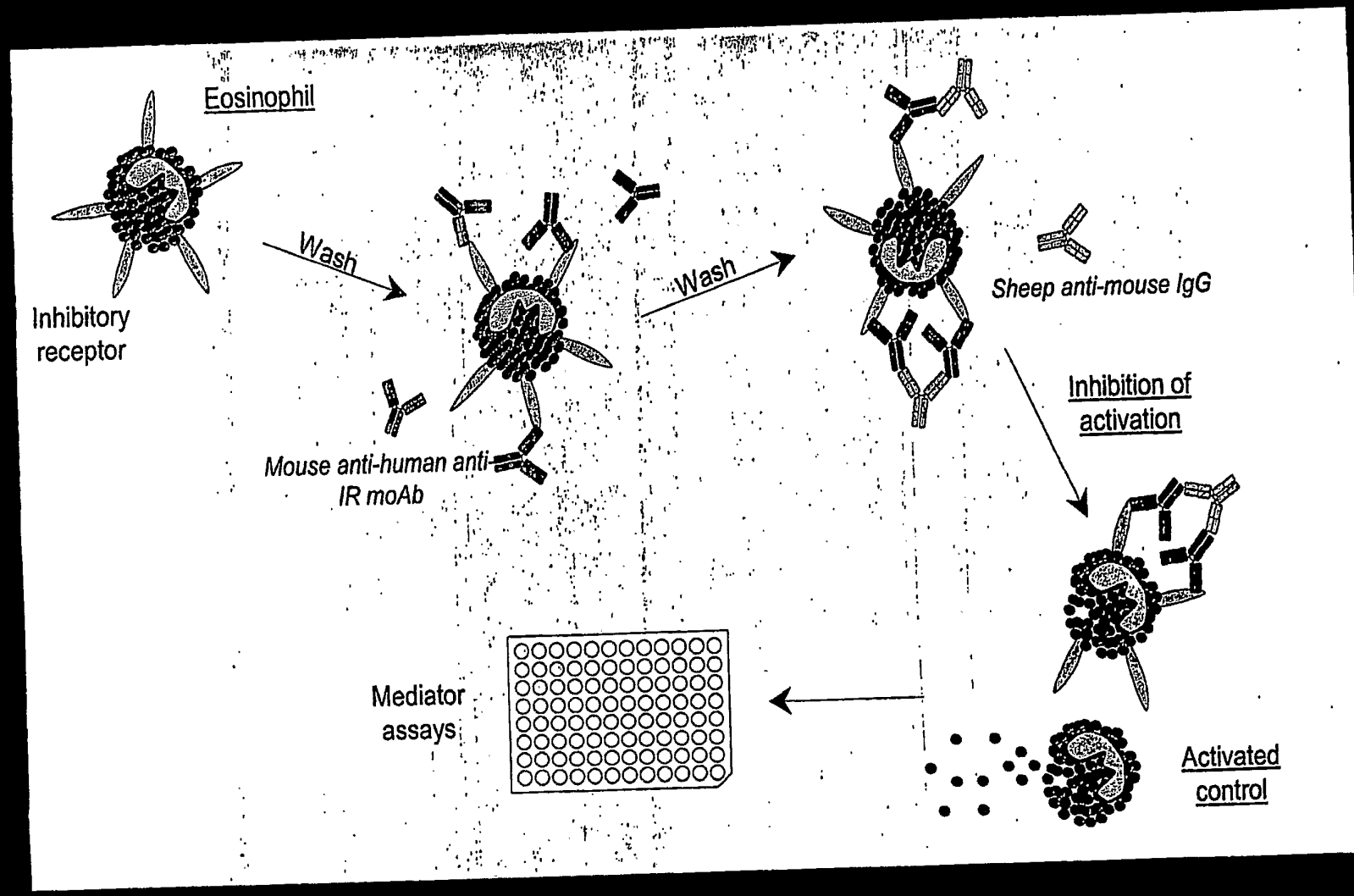
Known inhibitory receptors expressed on mast cells/eosinophils

 Human Mast cells	 Human Eosinophils
FcγRIIb **IRp60** **P75** Siglec-9	FcγRIIb **IRp60** **P75** **P140** LIR-3/ILT-5 Siglec-8 *, Siglec-10

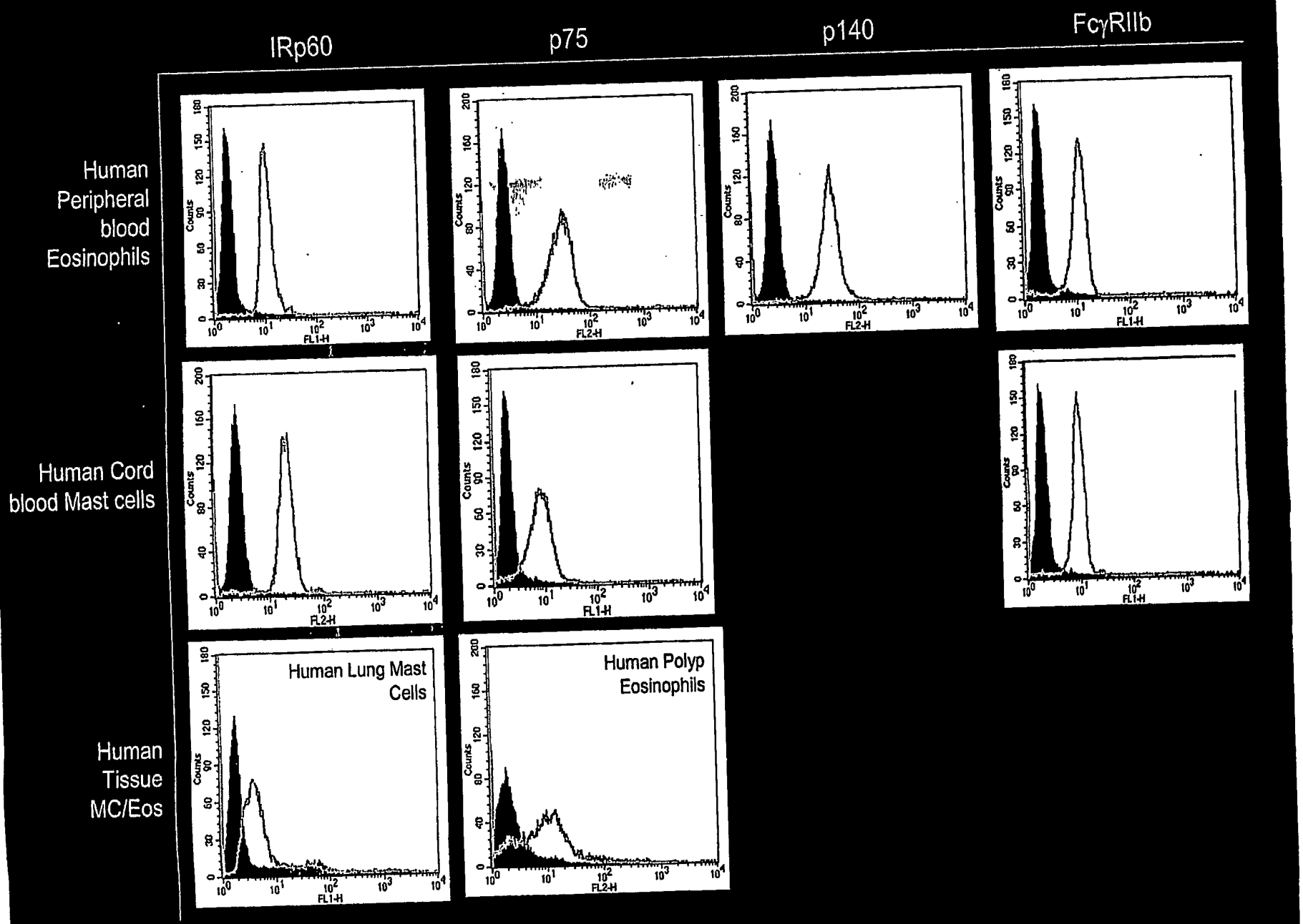
In yellow - discovered in our laboratory, unpublished data

*- Siglec-8 is an apoptosis-inducing receptor

In-vitro system for activation of inhibitory receptors developed in our laboratory

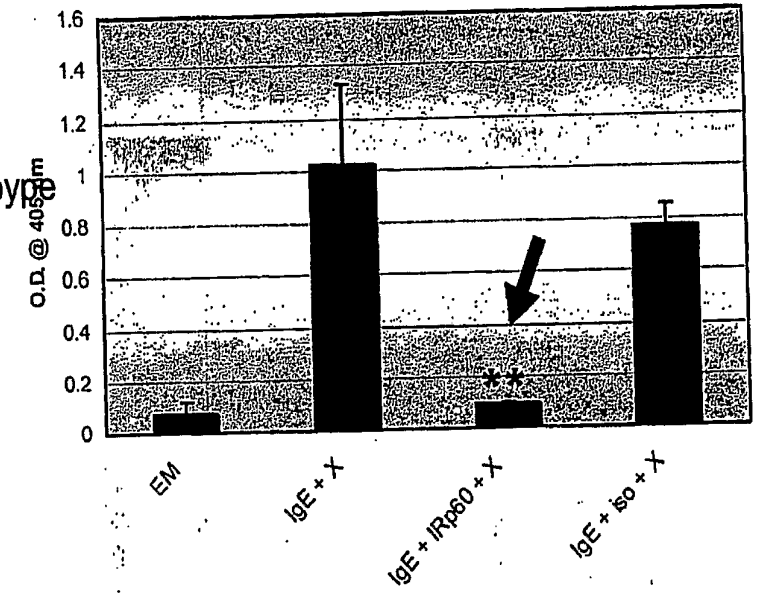


FACS screening of inhibitory receptor expression on human eosinophils and mast cells

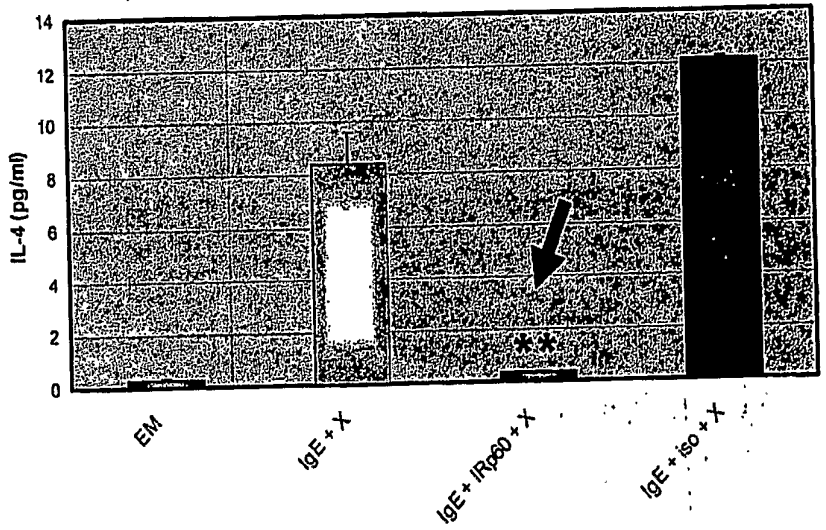


Inhibition of mast cell mediator release induced by IgE/IRp60 crosslinking

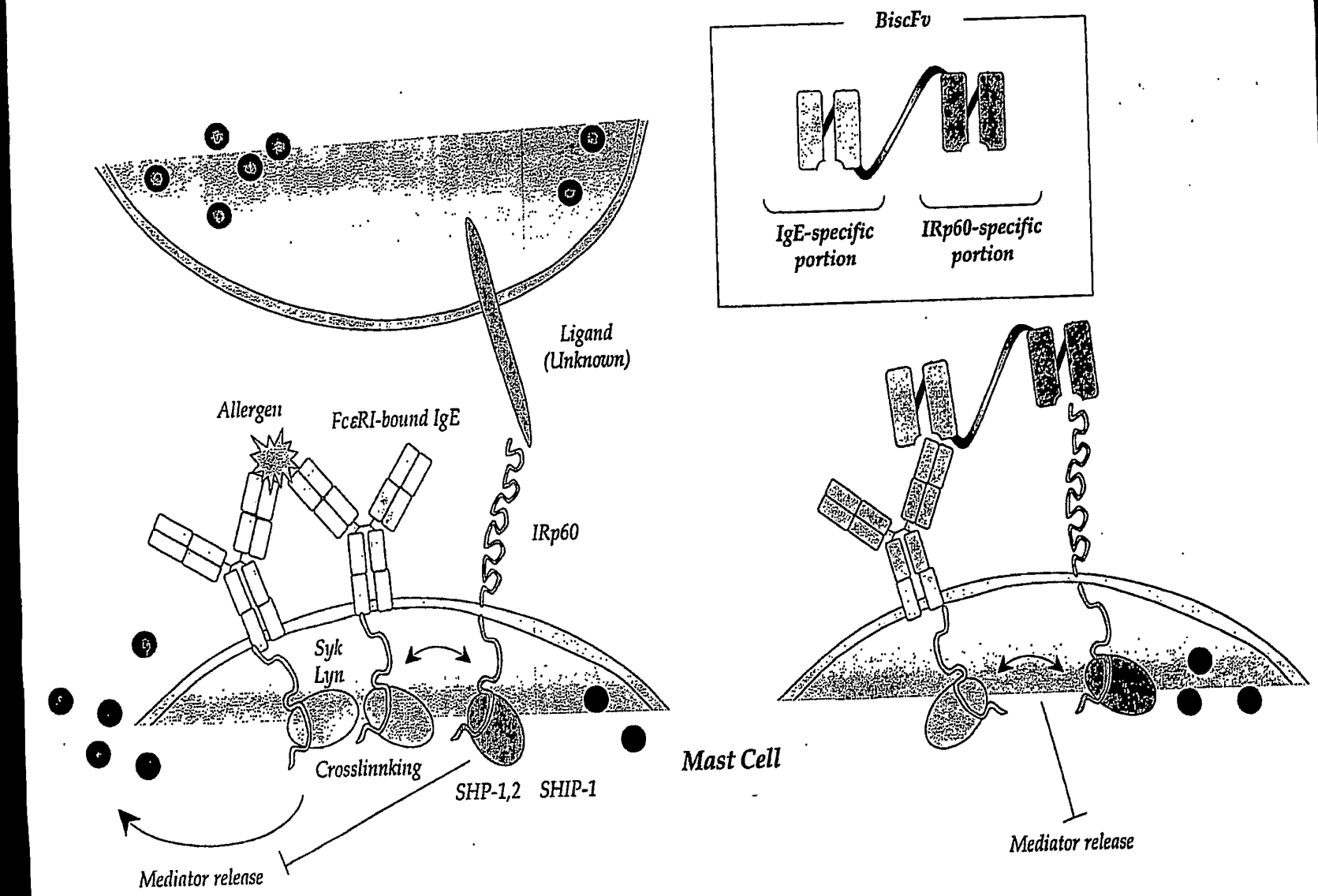
Beta-Hex (protoype enzyme)



IL-4



BiscFv (Bifunctional Single-Chain Fv fragment):

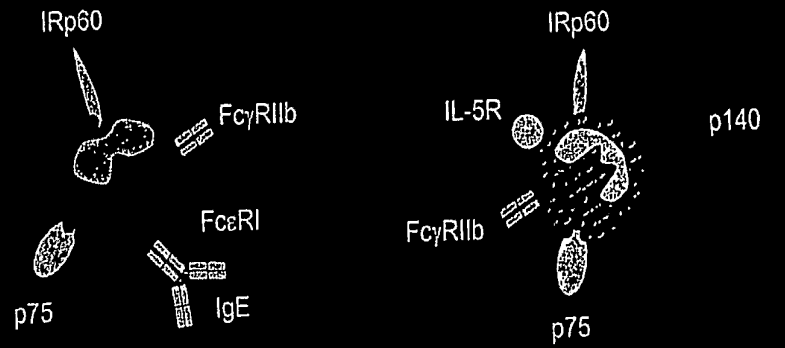


Aims and expected significance of the product

AIMS

1. To generate a mixture (cocktail) of bifunctional scFv (BiscFv) recognizing the following epitopes:

IgE / IRp60	***IL-5R / IRp60***
IgE / p75	***IL-5R / p75***
IgE / FcγRIIb	***IL-5R / p140***
	IL-5R / FcγRIIb

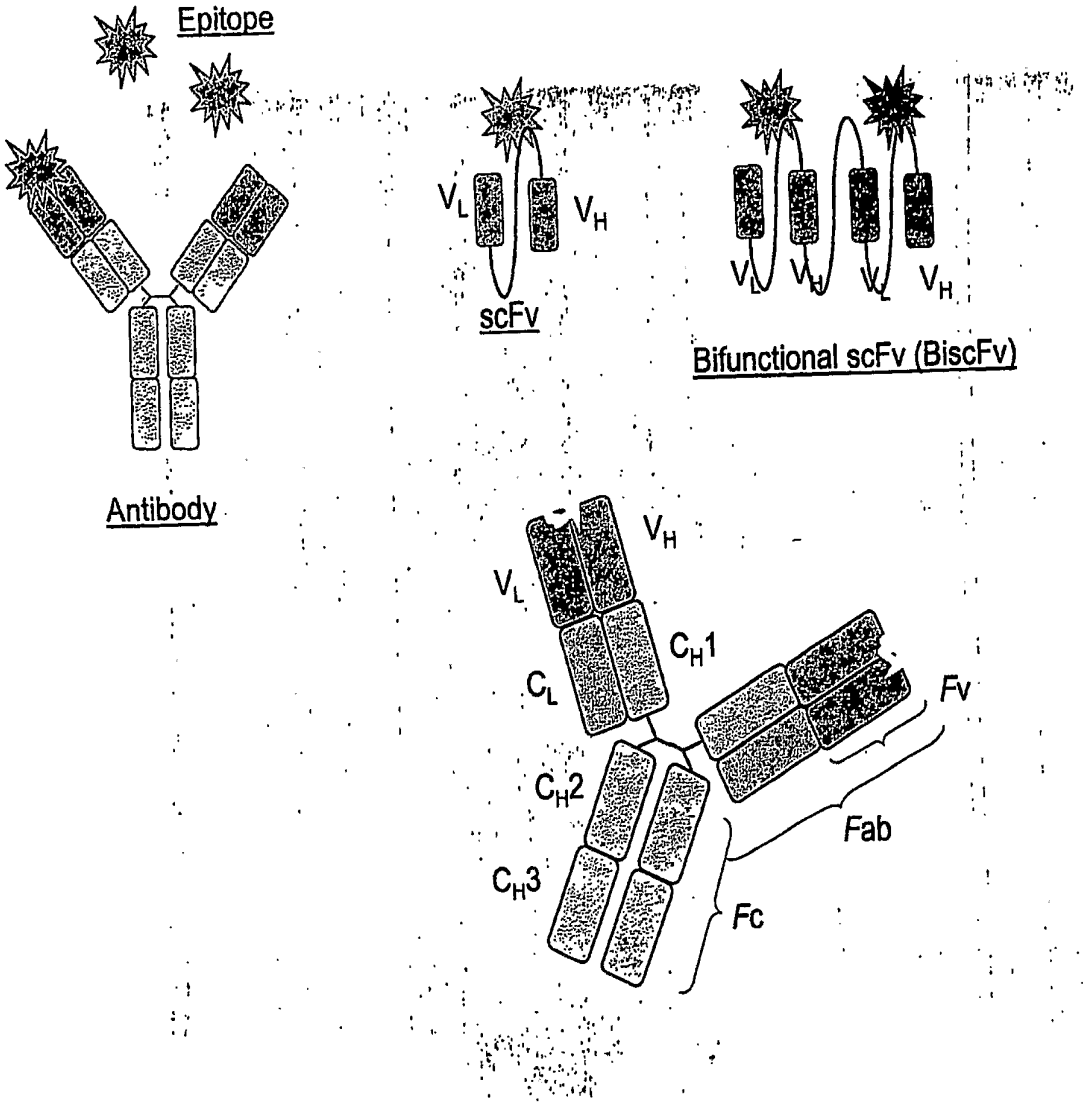


2. To test the *in-vitro* and *in-vivo* function and efficiency of the BiscFv cocktail.

Expected significance

1. The BiscFv cocktail will target the main effector cells of allergic disorders (i.e mast cells and eosinophils). Thus, simultaneously modulating their function via multiple pathways.
2. This novel approach for immune-modulation will provide a future platform for drug design in other pathological settings.

BiscFv (Bifunctional Single-Chain Fv fragment):



scFv:

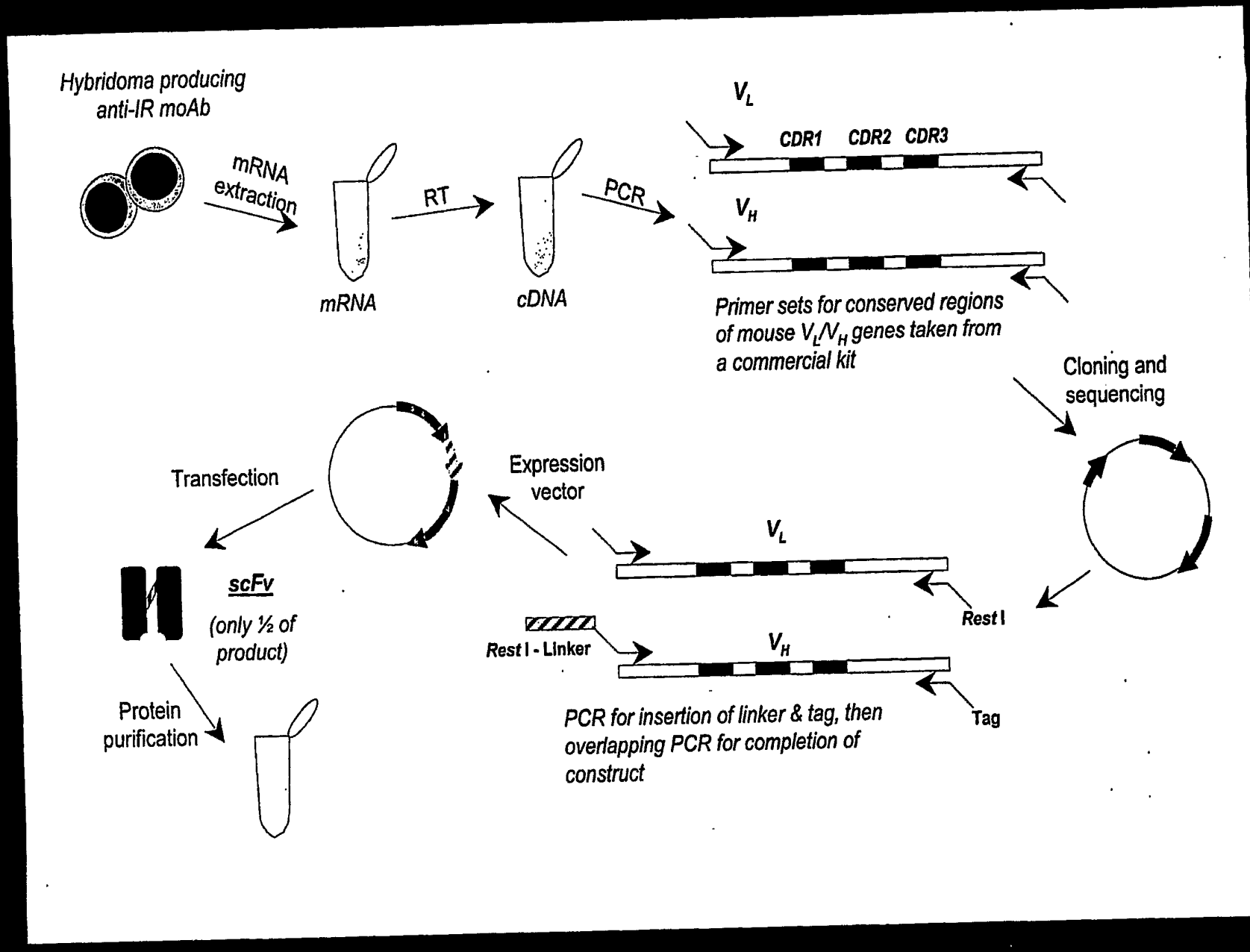
The basic-most unit of an antibody that retains recognition and binding functions

Linker:

3 repeats of **(Gly_4Ser)** motif.

Optimal for flexibility with minimal cross-bridges between cells

Generation of BiscFv (shown here only ½ of the final product)



BiscFv: predicted troubleshooting

	Predicted problem	Solution
1	*In-vitro* cell aggregation due to linker length	Generation of several linker lengths based on the $(Gly_4Ser)_3$ motif
2	Lack of *in-vivo* model (for several receptors)	1. Humanized SCID mice 2. Transgenic mice expressing human receptors
3	Clearance	Formulation for local administration

BiscFv: current project status

Stage	Components	Status
1	Hybridomas for IRp60, p75, p140, ILT-5, FcγRIIb, IgE, IL-5R	Yes: IRp60, p75, p140, ILT-5
2	Kits for amplification, cloning, expression and purification of BiscFv cocktail	No
3	*in-vivo* model	No
4		

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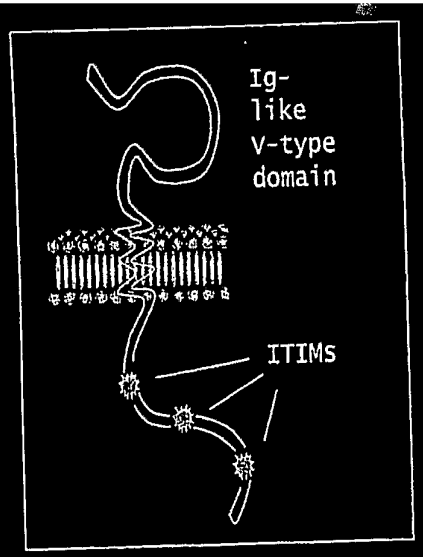
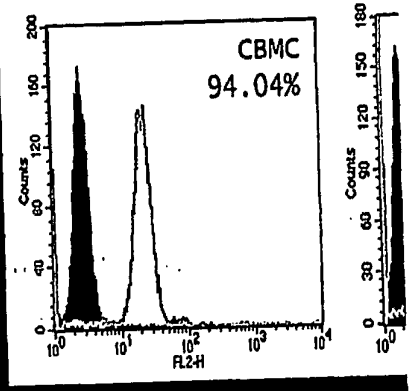
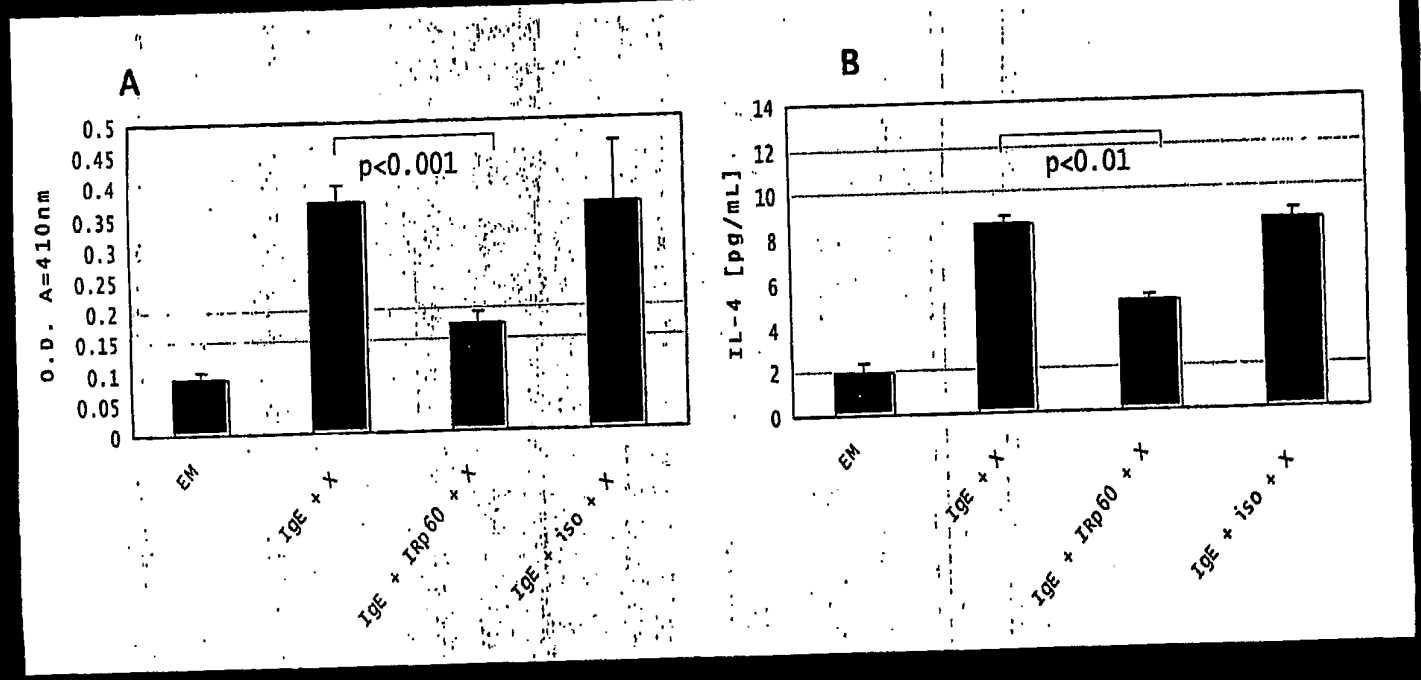


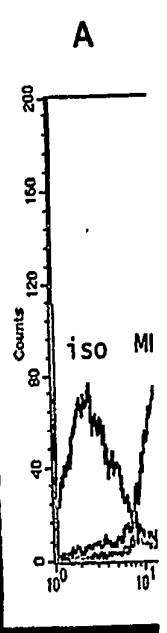
◀ FIGURE 1
A schematic representation of the putative IRp60 receptor. The receptor is characterized by a single Ig-like V-type domain, and 3 classical ITIM domains.



▲ FIGURE 2
IRp60 is expressed on human mast cells. IRp60 expression and WB were performed using F



▲ FIGURE 3
Cross-linking of FcεRI together with IRp60 inhibits β-hexosaminidase (A, 59.28±9.55% inhibition, n=4, p<0.001) and IL-4 (B, 64.43±2.91%, n=3, p<0.01) release from human cord blood derived mast cells. The effects are significant in comparison with an isotype control. EM, Enriched medium; X, Crosslinker; iso, isotype antibody.



▲ FIGURE 4
Incubation (10-100nM) expression, effect was

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